Protein Assay Protocol
For use with Pierce BCA™ Protein Assay Kit

Purpose, Harkins lab: Assay for total protein to be used in conjunction with NPY ELISA assay.

1. If unknown protein samples are stored in -80°C freezer, remove directly to ice for thawing.

Tip: Permit samples (in Eppendorf tubes) to rest on their sides on the surface of the ice until partially thawed. Once samples are almost thawed, place tubes vertically in ice. Thawing in this manner takes about one hour for ~1 mL of sample in a 1.5 or 2 mL Eppendorf tube.

2. Prepare 96-well plate.

Obtain a lidless 96-well plate and bring it to the bench. An old plate can be labeled "lid" and kept to use as a makeshift lid for the plate.

3. Prepare plate chart.

Obtain a clean plate chart. Label the chart with the contents of each well. You should have at least one blank well, which will be empty. Be certain to label the standard wells anti-alphabetically, so that moving from left to right, standard well are labeled I to A. You need a duplicate (2 replicates) of every well, including standards, samples, and any dilutions of samples that you make.

4. Prepare diluted albumin (BSA) standards.

*This chart is based on a stock 2.0 mg/mL albumin standard (BSA). It currently comes in the Pierce kit, in 1 mL ampules.

Directions:

a. Obtain 9, 1.5 or 2 mL Eppendorf tubes and label them A-I.
b. Add the appropriate amount of diluent to each tube.
   *The diluent is the solvent which your protein is stored in. In the Harkins lab, the diluent is usually water.
c. Add appropriate volume of BSA to Vial A.
d. Add appropriate volume of BSA to Vials B and C, briefly vortexing the tubes to mix the diluent and the BSA.
e. Transfer the appropriate amount of diluted BSA from Vial B to Vial D, and briefly vortex Vial D to mix the components. Continuing down the chart below (ie, in alphabetical order), continue to transfer diluted BSA to the next tube and briefly vortex it until Vial H has been prepared.

Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
A	0 µL	150 µL of Stock	2,000 µg/mL
B	62.5 µL	187.5 µL of Stock	1,500 µg/mL
C	162.5 µL	162.5 µL of Stock	1,000 µg/mL
D	87.5 µL	87.5 µL of vial B dilution	750 µg/mL
E	162.5 µL	162.5 µL of vial C dilution	500 µg/mL
F	162.5 µL	162.5 µL of via E dilution	250 µg/mL
G	162.5 µL	162.5 µL of vial F dilution	125 µg/mL
H	200 µL	50 µL of vial G dilution	25 µg/mL
I	200 µL	0 µL	0 µg/mL = Blank

5. Prepare the BCA™ Working Reagent (WR).

a. Determine the volumes of Reagent A and Reagent B needed.
   The working reagent (WR) should be composed of 50 parts Reagent A (from Pierce kit) and 1 part Reagent B (also from Pierce kit). 200 µL of WR is needed for each standard well and for

each sample well. In order to determine the amounts of Reagent A and Reagent B needed, use the following equation:

*Note: These calculations account for pipetting error.

(1) (Number of wells containing standard or unknowns*200 µL) + 200 µL = µL Reagent A
(2) µL Reagent A*0.02 = µL Reagent B

For example, if you were using 94 of the wells (leaving 2 for blank), you would need:
(1) (94*200 µL) + 200 µL = 19,000 µL Reagent A
(2) 19,000 µL Reagent A*0.02 = 380 µL Reagent B

b. Combine the calculated amounts of Reagent A and Reagent B in a conical tube which is large enough to contain the total volume. A 15 mL or 50 mL conical tube is most common. Briefly vortex to mix.

6. Load standard protein samples onto 96-well plate.

Pipette 25 µL of a standard protein sample into its designated well, as indicated on your plate chart. Once again, be certain to load standards beginning with I at the far left, and moving to A at the far right.

7. Load unknown protein samples onto the 96-well plate.

Once unknown protein samples are completely thawed, vortex sample very briefly and pipette 25 µL of each sample into its designated wells (normally, 2 wells, for replicates 1 and 2), as designated on your plate chart. It may be helpful to load samples in alphabetical or numerical order for additional clarity as to their locations.

8. Add WR to wells.

Add 200 µL WR (prepared in Step 4) to each well which contains either a standard or unknown protein sample. Proceed immediately to Step 9.

9. Incubate plate for 30 minutes.

Once WR has been added to protein-containing wells, cover the plate with a makeshift "lid" (an old plate) and place in rocking 37°C water bath. If necessary, add water to bring water level to approximately one-half the height of the plate. Place heavy rubber ring on plate to hold lid, and incubate for 30 minutes. Proceed immediately to Step 10.

10. Measure absorbance of plate.

After plate has incubated in 37°C water bath for 30 minutes, remove plate and proceed to plate reader (in Heather McArthur's lab).

a. Turn plate reader on.
b. Open plate reader and place plate inside, orienting the plate into the front right corner of the chamber.
c. Open computer program, "KC Junior."
d. Select the "Billy BSA" protocol.
e. Modify the protocol based on the contents of your plate. Be certain to designate any dilutions of samples that have been made, and to mark wells which are empty.
f. Run the protocol.
g. Print the calibration curve and the calculated sample concentrations.